Neo-nocardin, a New Antibiotic Produced by a New Species of Nocardia : 1. Taxonomical Studies of the Organism, Production and Extraction of the Antibiotic and its Antibacterial Activities

AUTHOR(S):

UESAKA, Ichiro

CITATION:

UESAKA, Ichiro. Neo-nocardin, a New Antibiotic Produced by a New Species of Nocardia : 1. Taxonomical Studies of the Organism, Production and Extraction of the Antibiotic and its Antibacterial Activities. Acta tuberculosea Japonica 1951, 1(1): 21-33

ISSUE DATE:

1951-06

URL:

http://hdl.handle.net/2433/51782

Neo-nocardin, a New Antibiotic Produced by a New Species of Nocardia.

1. Taxonomical Studies of the Organism, Production and Extraction of the Antibiotic and its Antibacterial Activities.

By

Ichiro UESAKA *

上坂一郎

(Received April 4, 1951)

Introduction.

Though there have been reported many antibiotics produced by streptomyces, reports on the antibiotic activities of nocardia, closely related genus of streptomyces, are comparatively rare [1)2)].

During my searches for new antibiotics produced by actinomycetes, I have found a new species of nocardia which produced a new antibiotic designated as neo-nocardin.

This paper describes the taxonomical studies of this strain, production of the active substance by the surface culture, antibacterial activity of the fermented broth, method of extraction, antibacterial activity and toxicity of crude extract, and finally the antagonisitic activity of the crude extract on tubercle bacillus in vitro.

1. Taxonomical Studies

In my collection of actinomycetes from soils I have found a strain of nocardia which showed a marked antibacterial activity and this strain was designated as strain No. A. 422.

*From the Bacteriological Laboratory (Chief: Prof. Saburo UYEDA) of the Tuberculosis Research Institute, Kyoto University

a). Morphological Studies

For the continuous observation of the organism the hanging drop method was used. Modified CZAPEK's agar or solution was used for the culture medium. Lactose was used for the carbon source in this medium, because lactose was superior to other sugars for the separation and dispersion of mycelia.

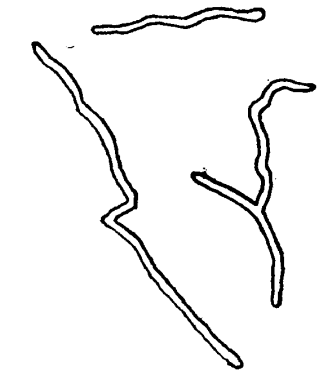


Fig. 1

1) Observation of fresh preparations

First day of cultivation (Fig. 1) : Mycelia are slightly curved. Cells are double-refracted. Usually two germ-tubes, about 0.8 to 18 microns in length.

Second (Fig. 2) and third (Fig. 3) day of cultivation: Mycelia grow further, branch abundantly and twine each other. Sometimes granules become visible in 3 days old culture.

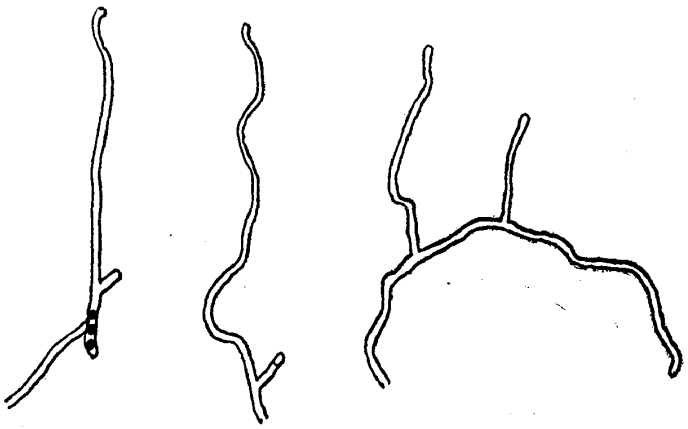


Fig. 2

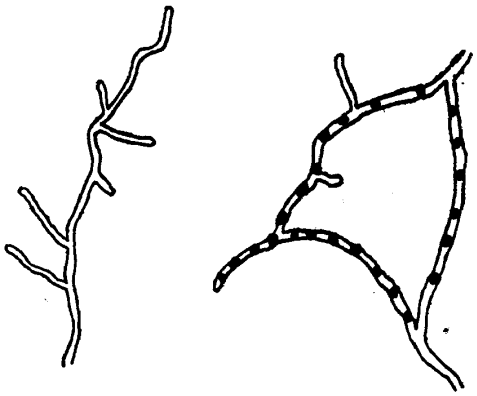


Fig. 3

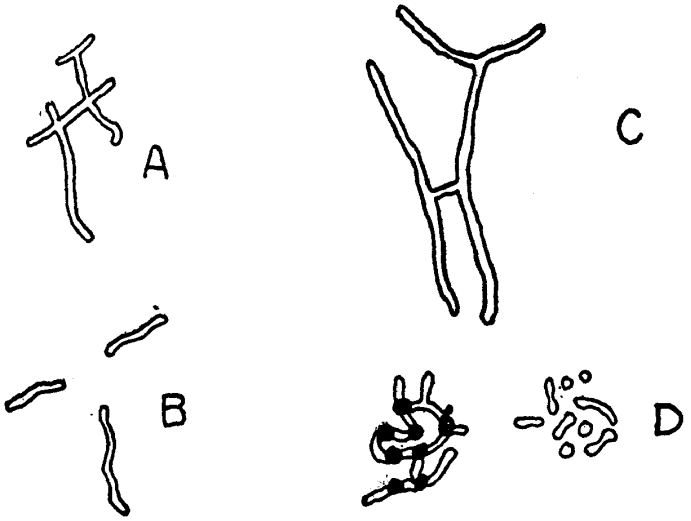


Fig. IV

Fifth and sixth day of cultivation and thereafter (Fig. 4) : Mycelia cling together and then separate into various forms of cells and disperse in the medium. In these cells double-refracted granules are present.

2) Obsevation of stained preparations

Methylene blue stain: Preparation of the 5th day culture was stained with methylene blue (Fig. 5). It was demonstrated that the mycelia in Fig. 4 were really made up of chains of rods or branched rods as shown in Fig. 5 (A) and (D). In the middle of these rods there exist condensed materials stained deep blue. Separated cells, about 0.8 by 1.3 microns, were also stained deep blue as shown in Fig. 5 (B).

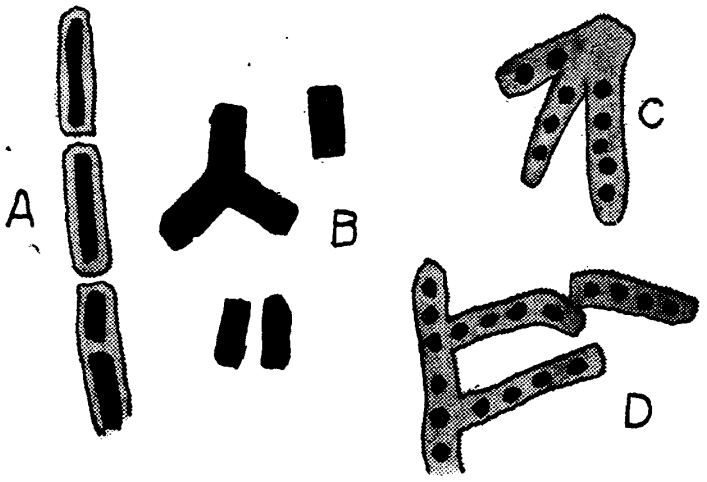


Fig. 5

Acid-fast stain: Though the mycelium was weak acid-fast, cells and their pre-stadium ··· Fig. 5 (A), (C) and (D) ··· were non acid-fast.

Gram stain: Gram-positive.

(*b*) *Physiological studies*

Separated cells were sown on various media and incubated at 37°C in order to establish a spectrum of nutrient sources and to observe the effects on certain different media The results are so comprehensive that only the characteristic properties will be described.

(1) Glucose agar: Abundant growth, at first yellowish brown, then gradually reddish brown. Aerial mycelium scant, white, at the margin of colonies. Red wine colored pigment.

(2) Synthetic agar: Pinkish white, punctiform growth. Grayish pink pigment.

(3) KRAINSKY's agar: Thin, pale yellow colonies, partly white punctiform aerial mycelium. Yellow pigment.

(4) Gelatin stab: Yellowish brown colony grows sinking in the medium, but no growth along the line of puncture. No aerial mycelium. Yellowish browh pigment. No liquefaction.

(5) Skimmed milk: No coagulation. Liquefaction after twe days. Brown pigment in the bottom of tube. The reaction gradually becomes alkaline.

(6) Blood agar: Numerous minute colonies. No aerial mycelium. Medium becomes black. No hemolysis.

(7) Egg media: Red colonies all over the surface of the medium. Gray aerial mycelium at the under margin of colonies. Slightly grayish pink

pigment.

(8) Starch is hydrolyzed.

(9) Nitrates are not reduced.

(10) Tyrosinase is not present.

(11) Nitrogen and carbon sources: As the nitrogen sources peptone and sodium nitrate are suitable. Galactose, mannose, lactose, maltose, dextrin, inulin, starch, glycogen, mannitol, and sorbitol stimulate the growth of the organism as the carbon sources.

(c) *Conclusion of the taxonomical studies.*

Morphological characteristics of No. 422 strain on the synthetic media (lactose as the carbon source) are as follows: In the early stages of growth this strain forms a long mycelium; but, after 5 to 6 days, the filaments break up into various forms of fragments, then into cylindrical short cells and I have never seen conidia in chains. Mycelia are weak acid-fast, but the cells and the fragments of mycelia are not acid-fast. From these characteristics this organism must belong to Nocardia according to the new nomenclature by WAKSMAN and HENRICI[3]. I could not find out, however, the species identical with this organism in the 33 species of genus Nocardia. Since this organism seems to belong to new species, I would like to name this strain as Nocardia Kuroishi n. sp..

2. *Production of the Active Substance by the Surface Culture*

(a) Factors influencing the antibacterial activity of culture fluid of A. 422 strain.

Sodium chloride suppressed the activity of the culture fluid. The inhibition of activity by salt increased with the increase in the concentration of salt. The activity of culture fluid was also found to decrease with the decrease of pH. The rabbit serum did not apparently influenced the activity. From these results it was found that the simplified broth (0.5 per cent meat extract and 0.5 per cent peptone, pH 7) was suitable for the assay of antibacterial properties of the culture fluid of this strain.

(b) Cultural conditions affecting the production of antibiotic substance (neo-nocardin).

(1) Addition of agar in the culture media: Addition of 0.2 per cent or 0.3 per cent agar in the glucose broth stimulated the growth and pigment production of this organism and increased its antibacterial properties.

(2) Intial pH of the culture media: The bacteriostatic effect of the culture fluid was investigated at the initial pH of 6.4 to 8.2. The maximum production of the antibiotic was obtained at initial pH of 7.0 to 7.6 and the activity rapidly decreased when the pH increased to over 7.8.

(3) Effect of ingredients of culture media on the antibacterial properties: Various carbon and nitrogen sources were investigated. As the carbon source glucose and starch have the same property for the prduction of neo-nocardin. Though there was no difference in the maximum yield of antibiotic, glycerol had the property to produce neo-nocardin earlier than glucose. Peptone was superior to sodium nitrate as the nitrogen source.

3. Antibacterial Properties of the Fermented Broth

The fermented broth was tested for the antibacterial activity against various bacteria (Table 1). The fermented broth inhibited the growth of gram-positive and gram-negative bacteria. The difference of susceptibility among the different strains in the same species of the test organisms is widely recognized in the case of penicillin and other antibiotics. This phenomenon was also observed in neo-nocardin (Table 2).

Table 1 Bacteriostatic activity of the fermented broth of No. A. 422 against various bacteria

	Bacteriostatic activity at dilution of
Staphylococcus aureus Terajima	1 : 160,000
Vibrio cholerae Inaba	1 : 3,200
Escherichia coli	1 : 3,200
Salmonella paratyphi A	1 : 3,200
Salmonella paratyphi B	1 : 100
Eberthella typhosa Boxhill	1 : 1,600
Eberthella typhosa H 901	1 : 1,600
Shigella dysenteriae Hanabusa	1 : 1,600
Shigella paradysenteriae Komagome B	1 : 100
Bacillus anthracis	1 : 1,600
Bacillus subtilis	1 : 100
Pseudomonas aeruginosa	< 1 : 10

Table 2 Difference of susceptibility among the different strains of staphylococci

Species	Strains	Penicillin susceptibility	Susceptibility to the culture fluid of No. A. 422
aureus	Wood	13.3※	1 : 64,000
	Terajima	16.3	1 : 40,000
	Yamase	17.9	1 : 8,000
	Heatley	16.3	1 . 2,000
	Hodo	20.4	1 : 1,000
	Kimura 1	12.1	1 : 100
albus	Noguchi	7.9	1 : 64,000
citreus	Shima 3	27.5	1 : 2,000

※ Numbers represent the length of inhibition zones in mm tested by the cup method.

4. *Method of Extraction*

(a) Stability of the active substance in the fermented broth:

The fermented broth was adjusted to various pH and heated at 100°C. Any loss of potency was not observed after 10 to 30 minutes preservation; after 60 minutes preservation, however, the potency decreased less than 1/10 compared with that of the original broth. On the contrary, the potency increased after 20 minutes preservation and such increase in potency was also observed when Eberthella typhosa or Escherichia coli was used in place of Staphylococcus aureus as the test organism.

(b) The active substance in the fermented broth was hardly dissolved in the solvents tested.

(c) Changes of antibacterial activity by the removal of mycelium:

For the extraction of the active substance from the fermented broth it is necessary to remove mycelium and agar from the broth.

The antibacterial activity of the filtrated broth was remarkably decreased by the removal of mycelium and agar independently of the methods of removal whether they were removed by centrifugation or by filtration with gauze. No decrease in potency was observed, however, when mycelium and agar were removed after the broth had been preserved at 100°C for 20 minutes.

(d) Adsorption of the active substance from the broth filtrate to carbon or to diatomaceous earth:

1) To activated carbon

After the culture filtrate was adjusted to various pH with HCl or NaOH and then treated with 1 per cent activated carbon and centrifuged, the potency of the supernatant was tested. Considerable amount of the active substance remained in the supernatant between pH 2 and 4. Adsorption of the active substance to the activated carbon was perfect between pH 5 and 7, while at pH 8 a small amount of the active substance remained in the supernatant.

2) To diatomaceous earth

The greatest amount of the active substance remained in the supernatant at pH 2. For this reason the broth filtrate wrs adjusted with HCl to pH 2 and treated with various concentrations of diatomaceous earth and centrifuged ; the potency of the supernatant was tested. The decrease of potency in supernatant was not observed by the treatment of 0.1 per cent diatomaceous earth, and mycelium and agar were entirely eliminated from the supernatant.

(e) Elution of the active substance from carbon

Elution into water, absolute ethanol or absolute methanol from the acti-

vated carbon was unsatisfactory, Elution into ethanol with or without HCl was then tested. The greatest amount of the active substance was eluted into 0.1 N HCl ethanol. Elution into 0.1 N HCl ethanol was, therefore, tested in comparison with elution into methanol with or without HCl. The greatest yield was gained by 0.1 N HCl methanol and the next was by 4/100 N HCl methanol and it was recognized that HCl ethanol was in general not a good eluent compared with HCl methanol. Among HCl methanols 4/100 N HCl methanol was superior to 0.1 N HCl methanol for the formation of precipitate by adding ether into the eluent containing the active substance.

Table 3 Adsorption of neo-nocardin to the diatomaceous earth

1)

Diatomaceous earth 1%		Color depth of fluid	Antistaphylococcal activity
Original broth (pH 8)		+6	1: 32,000
After the preservation at 100°C for 20 minutes		+6	1: 64,000
Supernatant after the adsorption to the diatomaceous earth from the heated broth	pH 2	+2	1: 2,000
	4	+3	1: 100
	6	+4	1: 100
	8	+6	1: 100

2)

Diatomaceous earth 0.5%	Antistaphylococcal activity
Original broth	1: 64,000
After the preservation at 100°C for 20 min	1: 128,000
Supernatant after the adsorption to the diatomaceous earth from the heated broth at pH 2.	1: 51,200

3)

Diatomaceous earth 0.2%	Antistaphylococcal activity
Original broth	1: 8000
After the preservation at 100°C for 20 min.	1: 8000
Supernatant after the adsorption to the diatomaceous earth from the heated broth at pH 2	1: 4000

4)

Diatomaceous earth 0.2%	Antistaphylococcal activity
Original broth	1: 4000
After the preservation at 100°C for 20 min.	1: 8000
Supernatant after the adsorption to the diatomaceous earth from the heated broth at pH 2.	1: 8000

From the above-mentioned results the whole process of extracting the active substance (neo-nocardin) from the broth to the crude hydrochloride can be summarized as Table 4.

Table 4 Extraction of neonocardin from the fermented broth

Fermented broth
↓
preserved at 100°C for 20 min., cooled, then filtered with gauze
↓
Filtrate
↓
pH 2 with HCl, added with 0.1% diatomaceous earth, centrifuged at 2500 r. p. m. for 20 to 30 min.
↓
Supernatant
↓
pH 5～6 with NaOH, added with 1% activated carbon, filtered.
↓
Sediment — Filtrate (colorless)
↓
Washed with water (1/10 volume of the broth)
↓
Washed with methanol (5 ml/g)
↓
Washed with ether
↓
Eluted into 1/100 N HCl methanol more than 3 times, centrifuged
↓
Eluate — Sediment
↓
precipitated with ether (3～4 volumes of eluate)
↓
dissolved into absolute methanol, centrifuged
↓
Supernatant — Sediment (Remains of carbon)
↓
Precipitated with ether (3～4 volumed of eluate)
↓
Crude neo-nocardin

The neo-noardin hydrochloride is hygroscopic, yellowish gray to grayish white powder.

5. *Antibacterial activity and toxicity of crude neo-nocardin hydrochloride*

(a) Antibacterial activity; The antibacterial activity of crude neo-nocardin hydrochloride was tested by the dilution method. The results are shown in Table 5. The antibacterial activity varied more or less with samples.

Table 5 Antibacterial activity of crude neo-nocardin

Test organism	Antibacterial activity
Staphylococcus aureus Terajima	1: 6,400,000
Vibrio cholerae Inaba	1: 1,600,000
Eberthella typhosa Boxhill	1: 800,000
Eberthella typhosa H 901	1: 800,000
Shigella dysenteriae Hanabusa	1: 800,000
Bacillus anthracis	1: 400,000
Bacillus subtilis	1: 100,000

Test medium: 0.5% Meat Extract + 0.5% Peptone

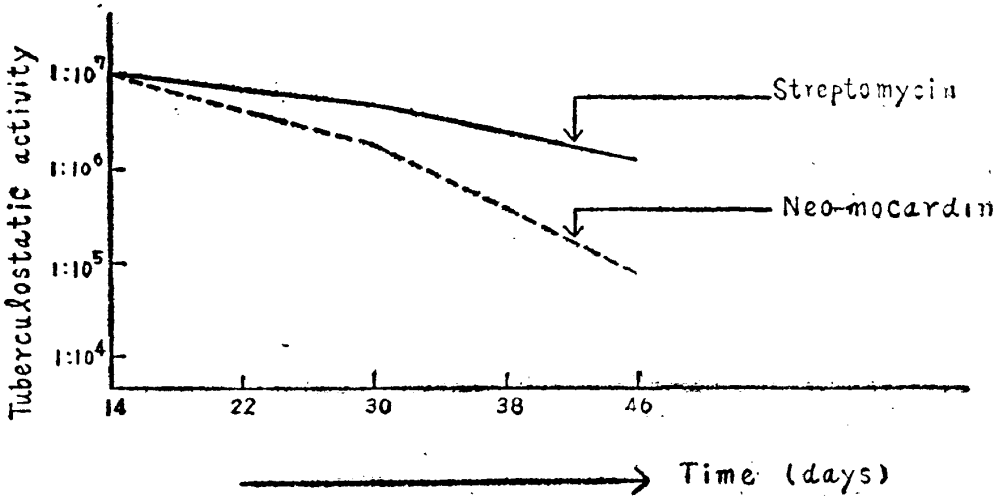
(b) Toxicity in mice: The crude neo-nocardin hydrochloride of 0.1, 0.5, 1.0 and 2.0 mg were dissolved in 0.5 ml of physiological NaCl solution and each 0.5 ml was injected into mice intraperitoneally (5 mice per one kind of concentration). They were observed for two weeks and all animals survived within these dosis of the antibiotic and any remarkable toxic effects were not observed.

6. *Antagonistic Activity of Crude Neo-nocardin Hydrochloride on Tubercle Bacilli In Vitro*

The antagonistic activity of crude neo-nocardin hydrochloride on tubercle bacilli was investigated. It is widely known there are antibiotics of which activities decrease when these are preserved at 37°C.

As seen in Fig. 6, though the activities of streptomycin and neo-nocardin

Fig. 6 Changes of antibiotic activities of neo-nocardin and streptomycin on tubercle bacilli by the preservation at 37°C



on tubercle bacilli in Kirchner media were the same after 14 days preservation, their activities gradually decrease with progressing time. But the decrease of activity of neo-nocardin was more rapid than that of streptomycin and after 46 days incubation the activity of the former was only about 1/20 as compared with that of the latter. From the above-mentioned results is it reasonable to consider that neo-nocardin might also be ineffective in vivo? I never think so.

Drugs are as a rule administered everyday for patients or for experimentally infected animals so far as any toxic effect is not found. It is usually out of question whether the activity of drug might be decreased at the next day of administration or not. The drug should maintain its activity at least during the day of administration, and on the next day the growth of the bacilli may be inhibited by the administration of the day. In this reason I think it is worthless to determine the activity of drugs after 5 or 6 weeks incubation as it is usually practised by the antituberculous agents. I think it is necessary to devise new methods which are able to determine the activity at least a few days after incubation. According to this principle I have devised two methods of determining the activities of antituberculous agents in a few days, and using these methods I have cpmpared the activity of neo-nocardin on tubercle bacilli with that of streptomycin.

(a) Microscopic method: The agents ··· streptomycin and crude neo-nocardin ··· dissolved in distilled water. Subsequent dilutions were made directly into 1.0 ml of Kirchner media. Each tubes were inoculated with saline suspension of tubercle bacilli. The control tubes containing no agents but inoculated with tubercle bacilli were prepared. All these tubes were incubated at 37°C. From the next day on one control tube a day was taken out from the incubator and the supernatant was removed without agitating the content. The remaining 0.2 to 0.3 ml of fluid was soaked up and dropped on several pieces of slide glasses. These glasses were dried, fixed and stained in due form and then examined microscopically. On this occasion I examined at first by dry system (80 X) and if the bacterial growth was not found I examined by oil immersion system (800 X). If the initial growth of the bacilli, serpentine growth specific to this organism, was recognized, I treated

Table 6 Microscopic method

1) Dry system (80X Magnification, after 7 days)

Antibiotics \ Dilutions	2	4	8	16	32	64	128	256	512	1024	2048	×10⁴	Control
Streptomycin	−	−	−	−	−	−	−	−	+	+	+		+
Neo-nocardin	−	−	−	−	−	−	−	+	+	+	+		

Table 7 Subcultivation method.

Subcultured on egg media after		2	4	8	16	32	64	128	256	512	1024	2048 ×10⁴	Control 1	Control 2
day (s) 1	Streptomycin	12	3	18	17	50	78	68	59	48	ca. 130	ca. 130 ⊕	ca. 140	ca. 120
	Neo-nocardin	38	46	29	39	46	83	63	79	45	111	ca. 120	ca. 120	ca. 130
3	Streptomycin	6	1	1	0	4	18	85	82	ca. 130	ca. 140	ca. 140	ca. 200	
	Neo-nocardin	3	22	33	55	22	8	89	78	ca. 120	ca. 150	ca. 150	ca. 150	
3	Streptomycin	5	0	2	3	1	3	C	35	ca. 200	ca. 200	>200	>200	
	Neo-nocardin	5	12	17	48	40	ca. 200	ca. 150	ca. 200	>200	ca. 200	>200	>200	

Note: (1) 0.04 ml of saline suspension of tubercle bacilli (0.05 mg/ml) was inoculated into each tubes of Kirchner media containing serially diluted antibiotics and after 1, 3 and 5 days of incubation the bacilli were subcultivated on egg media.

(2) Control 1 is the one which was subcultivated from tubes containing no antibiotics. Control 2 is the one which was subcultivated from tubes containing no antibiotics on the day the bacilli were inoculated in Kirchner media.

(3) C indicates contamination.

(4) Numbers⊕ represent colony counts on egg media.

2) Oil immersion system (800 X Magnification, after 3 days)

Antibiotics \ Dilutions	2	4	8	16	32	64	128	256	512	1024	2048 ×10⁴	Control
Streptomycin	−	−	−	−	−	−	−	−	−	+	+	+
Neo-nocardin	−	−	−	−	−	−	−	−	−	+	+	

all the tubes containing agents in the same way as I had done in the control tube and examined microscopically. As seen in Table 6 the bacterial growth was observed after 3 days incubation by the oil immersion system, and the antibiotic activities of streptomycin and neo-nocardin were the same. But, if the dry system was used it was necessary to find out the bacterial growth for 7 days, and on this occasion streptomycin inhibited the bacterial growth at the concentration of 1 : 2,560,000 and neo-nocardin, at 1 : 1,280,000.

(b) Subcultivation method: The agents were dissolved in distilled water and subsequent dilutions were made directly into Kirchner media. Each tubes were inoculated with saline suspension of tubercle bacilli. All the tubes were incubated at 37°C. From the next day of incubation subcultivation onto egg media was made from all the tubes. Colonies grown on the egg media counted. As seen in Table 7 the antagonistic activity of streptomycin and neo-nocardin after one and three days incubation were the same, but after five days the activity of streptomycin was a little greater than that of neo-nocardin.

(c) Conclusion of the studies on the in vitro activity of neo-nocardin against tubercle bacilli: In the in vitro screening test of antituberculous agents against tubercle bacilli the determination of activity should be done as early as possible. According to this principle I have devised two new methods of testing the activity against tubercle bacilli in vitro. By these two new methods I have compared the antituberculous activity of neo-nocardin with that of streptomycin. I have found that the two antibiotics have the almost equal activity against tubercle bacilli, if the determination of activity has been done as early as possible. This result indicates the new antibiotic, neo-nocardin, is available to the treatment of experimental tuberculosis.

Summary

1. I have found a new strain of nocardia which produced a new antibiotic designated as neo-nocardin. By the taxonomical studies of this strain it was found to belong to a new species of nocardia and was designated tentatively as Nocardia kuroishi n. sp. .

Production of neo-nocardin by the surface culture of N. kuroishi A. 422 was investigated. Antibacterial activity of the fermented broth was suppressed by NaCl and increased with the increase in pH of test media. Addition of agar in the culture media increases the growth of the organism and the production of neo-nocardin. Glucose broth is a good media for the production of neo-nocardin, if 0.2 or 0.3 per cent agar is added to the media.

3. Method of extraction of neo-nocardin from the fermented broth was

investigated. After the adsorption of the antibiotic to the activated carbon it was eluted into 4/100 N HCl methanol and then precipitated with ether. The crude neo-nocardin hydrochloride as well as fermented broth of this organism were active against gram-positive and gram-negative bacteria.

4. In mice injected with 2 mg of crude neo-nocardin hydrochloride intraperitoneally any remarkable toxic effects were not observed.

5. Crude neo-nocardin hydrochloride has the equal activity as streptomycin against tubercle bacilli in vitro.

Acknowledgement

The author is indebted to Prof. Saburo UYEDA for his kind directions throughout the work

References.

1) A. D. GARDNER & E. CHAIN Proactinomycin: a "bacteriostatic" produced by a species of Proactionmyces. Brit. J. Exp. Path., 23: 123-127, 1942 cited in S. A. WAKSMAN Microbial antagonisms and antibiotic substances. The Commonwealth Fund New York 1945

2) E. W. EMMART A new tuberculostatic antibiotic produced by Nocardia. A preliminary report. Amer. Rev. Tuberc., 55: 4, 316-333, Oct., 1947 E. W. EMMART, R. E. KISSLING & T. H. STARK The tuberculostatic action of Nocardia extracts in white mice. J. Bact., 57: 5, 509-514, May, 1949

3) WAKSMAN & HENRICI BERGEY's Manual of Determinative Bacteriology, 6th ed., 1948